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Development of a One-Step Multiplex PCR Assay for Differential Detection of Major *Mycobacterium* Species

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ABSTRACT The prevalence of tuberculosis continues to be high, and nontuberculous mycobacterial (NTM) infection has also emerged worldwide. Moreover, differential and accurate identification of mycobacteria to the species or subspecies level is an unmet clinical need. Here, we developed a one-step multiplex PCR assay using whole-genome analysis and bioinformatics to identify novel molecular targets. The aims of this assay were to (i) discriminate between the *Mycobacterium tuberculosis* complex (MTBC) and NTM using *rv0577* or RD750, (ii) differentiate *M. tuberculosis* (*M. tuberculosis*) from MTBC using RD9, (iii) selectively identify the widespread *M. tuberculosis* Beijing genotype by targeting *mtbk_20680*, and (iv) simultaneously detect five clinically important NTM (*M. avium*, *M. intracellulare*, *M. abscessus*, *M. massiliense*, and *M. kansasii*) by targeting IS*1311*, DT1, *mass_3210*, and *mkan_rs12360*. An initial evaluation of the multiplex PCR assay using reference strains demonstrated 100% specificity for the targeted *Mycobacterium* species. Analytical sensitivity ranged from 1 to 10 pg for extracted DNA and was 10^3 and 10^4 CFU for pure cultures and nonhomogenized artificial sputum cultures, respectively, of the targeted species. The accuracy of the multiplex PCR assay was further evaluated using 55 reference strains and 94 mycobacterial clinical isolates. Spoligotyping, multilocus sequence analysis, and a commercial real-time PCR assay were employed as standard assays to evaluate the multiplex PCR assay with clinical *M. tuberculosis* and NTM isolates. The PCR assay displayed 100% identification agreement with the standard assays. Our multiplex PCR assay is a simple, convenient, and reliable technique for differential identification of MTBC, *M. tuberculosis*, *M. tuberculosis* Beijing genotype, and major NTM species.

KEYWORDS *Mycobacterium tuberculosis*, Beijing genotype, nontuberculous mycobacteria, *Mycobacterium avium* complex, *Mycobacterium abscessus* complex, *Mycobacterium kansasii*, multiplex PCR

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The genus *Mycobacterium* consists of more than 170 species/subspecies (http://www.bacterio.net/mycobacterium.html). Differential identification of the major mycobacterial species associated with human disease is important for practical use in clinical laboratories. Tuberculosis (TB), caused by members of the *Mycobacterium tuberculosis* complex (MTBC) and mainly by *M. tuberculosis*, has been a significant illness and cause of death among humans for centuries (1). *M. tuberculosis* strains cluster into seven major lineages, each associated with a specific geographic location (2). In particular, the *M. tuberculosis* Beijing genotype family has been significantly associated with major TB outbreaks over the past decade throughout the world (3). The *M. tuber-*

culosis Beijing family has been isolated in at least 13% of all MTBC cases around the world (3, 4) and is especially prevalent among people living in the former Soviet Union (35%) (5), South Africa (17%), and East Asian countries, including China, Japan, and Korea (64%) (6). The *M. tuberculosis* Beijing family has drawn attention because of its hypervirulent phenotype, which has been correlated with higher rates of relapse (7, 8) and treatment failure, the latter of which could be strongly associated with the rapid spread of multidrug-resistant *M. tuberculosis* (MDR *M. tuberculosis*). The World Health Organization has reported that the proportion of *M. tuberculosis* strains with multidrug resistance is over 4% worldwide. Although the MDR proportion of *M. tuberculosis* strains belonging to the Beijing family differs according to the regions, the average proportion of strains of the Beijing family with multidrug resistance is relatively high compared to other lineages (3, 9, 10). Moreover, in some countries where the *M. tuberculosis* Beijing family is highly dominant, low protective efficacy of the *M. bovis* BCG (bacillus Calmette-Guérin) vaccine has been reported (11). Consequently, as the isolation rate of strains of the *M. tuberculosis* Beijing family increases worldwide, epidemiological control of TB will face even greater challenges (2). Therefore, identification of the isolates of this family is an important part of TB control efforts.

Nontuberculous mycobacteria (NTM) have also become recognized as important human pathogens, with the incidence and prevalence of NTM disease continuing to increase worldwide (12). Although the major causative organisms of NTM disease differ by country and/or region, *M. avium* complex (MAC) members such as *M. avium* and *M. intracellulare*, *M. abscessus* complex (MABC) members such as *M. abscessus* subsp. *abscessus* (here referred to as *M. abscessus*) and *M. abscessus* subsp. *massiliense* (here referred to as *M. massiliense*), and *M. kansasii* are the most important NTM species and subspecies and are frequently isolated from patients with NTM disease (13). Differentiating between patients with NTM disease and patients with TB is very important because the therapeutic regimens for these two types of infections differ and, in contrast to TB cases, it is unnecessary to track down contacts of patients with NTM disease (12, 14). In addition, accurate identification of NTM species or subspecies is important because antibiotic susceptibility and treatment outcomes differ according to the etiologic NTM organism (12, 14).

For these reasons, it is both clinically and epidemiologically important to accurately identify human-disease-associated mycobacterial isolates to the species or lineage level for the appropriate clinical management of patients and to ensure public health. Thus, the development of a method for achieving an accurate and prompt identification of major *Mycobacterium* species has been of great interest in the clinical setting.

The primary goal of our study was to develop and optimize a single-step multiplex PCR assay capable of differentiating the major *Mycobacterium* species causing human disease. Our optimized multiplex PCR assay is composed of eight primer sets for identifying MTBC species, Beijing family strains and non-Beijing strains of *M. tuberculosis*, and five major NTM species (*M. avium*, *M. intracellulare*, *M. abscessus*, *M. massiliense*, and *M. kansasii*) and will provide a reliable and rapid detection method for laboratory diagnosis to employ in the clinical setting.

RESULTS

Selection of target genes in major mycobacterial pathogens for development of the multiplex PCR assay. In this study, we designed primer sets to identify major mycobacterial species or strains in the following order: (i) to discriminate panmycobacterial from nonmycobacterial species; (ii) to discriminate between MTBC and NTM species in mycobacteria; (iii) to differentiate *M. tuberculosis* from other MTBC species as well as identify the five major NTM species and subspecies (*M. avium*, *M. intracellulare*, *M. abscessus*, *M. massiliense*, and *M. kansasii*); and (iv) to identify *M. tuberculosis* Beijing family strains.

Eight primer sets were used to develop the multiplex PCR assay, with each primer set targeting a specific locus (Table 1, sets 1 to 8). We identified two primer sets (*mtbk_20680* and *mass_3210*) by comparative genome analysis (Fig. 1); the remaining

TABLE 1 Primers used in the multiplex PCR assay according to target *Mycobacterium* species

Set	Genetic target	Primer sequences[a] (forward and reverse)	Target organism(s)	Expected product size (bp)
1	16S rRNA gene	5′ GAGATACTCGAGTGGCGAAC 3′ 5′ CAACGCGACAAACCACCTAC 3′	All mycobacterial species	506
2	*rv0577*	5′ ATGCCCAAGAGAAGCGAATACA 3′ 5′ AATGTCAGCCGGTTCCGCAA 3′	All *M. tuberculosis* complex	705
3	RD9	5′ GTGTAGGTCAGCCCCATCC 3′ 5′ GTAAGCGCGTGGTGTGGA 3′	*M. tuberculosis*	369
4	*mtbk_20680*	5′ TTATGCCAGAAATACACCCGCG 3′ 5′ AATCGCGGGCTTGTGGCTAC 3′	*M. tuberculosis* Beijing family	231
5	IS*1311*	5′ TCGATCAGTGCTTGTTCGCG 3′ 5′ CGATGGTGTCGAGTTGCTCT 3′	*M. avium* complex	600
6	DT1	5′ AAGGTGAGCCCAGCTTTGAACTCCA 3′ 5′ GCGCTTCATTCGCGATCATCAGGTG 3′	*M. intracellulare*	106
7	*mass_3210*	5′ GCTTGTTCCCGGTGCCACAC 3′ 5′ GGAGCGCGATGCGTCAGGAC 3′	*M. abscessus* *M. massiliense*	310 1145
8	*mkan_rs12360*	5′ ACAAACGGTGTGTCGCAATGTGCCA 3′ 5′ TGTCGAGCAGACGTTCCAGGACGGT 3′	*M. kansasii*	199
9	RD750[b]	5′ TTGCACAGCTTGGCGACGAAT 3′ 5′ ATGGCCTCGTGTCTCCCAAAACT 3′	*M. tuberculosis* complex (excluding Central Asia strain)	865

[a]All primers were newly designed for this study, with the exception of the RD9 primer pair (17).
[b]Primer sets 1 to 8 were used for the first multiplex PCR assay; set 9 was used in the second multiplex PCR assay for optimization of CAS detection.

specific primer sets, including the panmycobacterial 16S rRNA gene set and the sets for *rv0577*, RD9, IS*1311*, DT1, and *mkan_rs12360*, were constructed based on previous publications and reference sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/), followed by bioinformatics analysis as described in Materials and Methods.

16S rRNA gene (for detection of all mycobacteria). For pandetection of the genus *Mycobacterium*, we selected a consensus nonpolymorphic region of the 16S rRNA gene from six target mycobacterial species or subspecies (*M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. abscessus*, *M. massiliense*, and *M. kansasii*) (15). This consensus region can be used as a positive control when testing for the presence of mycobacteria with the multiplex PCR.

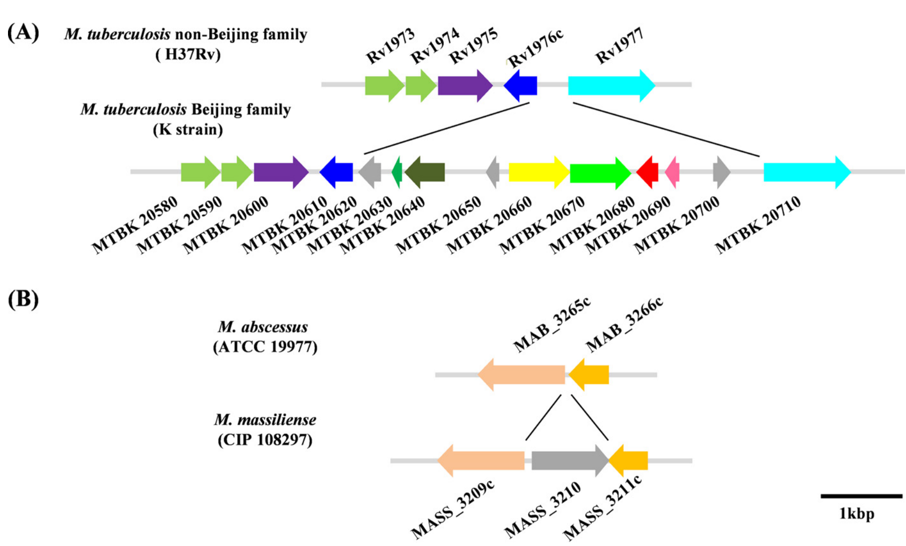

FIG 1 Specific genes conserved in the *M. tuberculosis* Beijing family (A) and *M. massiliense* (B). (A) We compared the *M. tuberculosis* H37Rv (non-Beijing family, GenBank accession no. NC_000962) genome with the *M. tuberculosis* strain K (Beijing family, GenBank accession no. CP007803.1) genome. The region from *mtbk_20620* to *mtbk_20700* was an inserted gene cluster specific to *M. tuberculosis* K. (B) On alignment of the complete genomes of *M. abscessus* (GenBank accession no. CU458896) and *M. massiliense* (GenBank accession no. NZ_JRMG00000000.1), one gene (*mass_3210*) that could discriminate between *M. abscessus* and *M. massiliense* was found.

***rv0577* (for discrimination of MTBC from NTM).** IS*6110*, a marker commonly used to define MTBC, was reported to be absent from some *M. tuberculosis* strains and to be present in some NTM strains (16). Therefore, *rv0577* was chosen as the genotypic maker for the MTBC. *rv0577* is an MTBC-restricted gene and was thus used to discriminate MTBC from NTM species (15).

RD9 (for identification of *M. tuberculosis* in MTBC). RD9 corresponds to the intergenic region between *rv2072*c and *rv2073*c. This sequence is unique to *M. tuberculosis* and is absent from all other MTBC members (except for *M. canettii*) and from NTM species. Thus, selective amplification of this region is considered to be indicative of *M. tuberculosis* (17).

***mtbk_20680* (for identification of strains of the Beijing family of *M. tuberculosis*).** Based on a comparative genomic analysis of *M. tuberculosis* K (*M. tuberculosis* Beijing family) and H37Rv (*M. tuberculosis* non-Beijing family), five *M. tuberculosis* K-specific gene clusters were identified (data not shown). One of these regions was completely conserved in all reference strains of the *M. tuberculosis* Beijing family but was absent from *M. tuberculosis* non-Beijing family strains. This region, which corresponds to *mtbk_20620* to *mtbk_20700* (Fig. 1A), contains nine genes, including the proline-glutamic acid family gene *mtbk_20640*, encoding a mycobacterium-specific virulence factor, the *tuf* gene *mtbk_20680*, encoding the iron-regulated elongation factor Tu, and genes encoding various hypothetical proteins. Based on an alignment of all *M. tuberculosis* Beijing clinical isolate genome sequences, a section of *mtbk_20680*, which was the most highly conserved sequence without polymorphisms in this region, was selected as the specific molecular target to identify the *M. tuberculosis* Beijing family.

IS*1311* and DT1 (for identification of *M. avium* and *M. intracellulare*). IS*1311* and DT1 are insertion elements harboring transposase genes in *M. avium* and *M. intracellulare*, respectively. Although insertion elements can be unstable, IS*1311* and DT1 are consistently associated with *M. avium* and *M. intracellulare* (18). Thus, selective amplification of these regions is considered to indicate *M. avium* and *M. intracellulare*, respectively, although some exceptions exist.

***mass_3210* (for identification of *M. abscessus* and *M. massiliense*).** Using whole-genome sequence analysis, we previously discovered specific genes that potentially discriminate between MABC species (19). Among these specific genes, *mass_3210* (879 bp) is specific to *M. massiliense* and is located between *mass_3209* and *mass_3211*, which correspond to *mab_3265*c and *mab_3266*c, respectively, in the *M. abscessus* genome. This insertion gene region can be used to differentiate *M. abscessus* from *M. massiliense* by designing primers to sites flanking *mass_3210*, i.e., to positions in *mab_3265*c/*mass_3209* and *mab_3266*c/*mass_3211* (Fig. 1B).

***mkan_rs12360* (for identification of *M. kansasii*).** Before the whole-genome sequence of *M. kansasii* was published, p6123 was used as a specific DNA probe to identify *M. kansasii*. In addition, this sequence has also been used as a marker to detect the major types I and II of *M. kansasii*, which cause human disease (20). The alignment analysis in our current study revealed that the *M. kansasii*-specific sequence cloned into p6123 corresponds to the *mkan_rs12360* region. Thus, in this study, the *M. kansasii*-specific primers were designed based on the most highly conserved sequence without polymorphisms in *mkan_rs12360*.

Multiplex PCR design and interpretation. The assay utilizes an eight-target multiplex PCR, and the presence of specific amplicons is readily visualized on 6% acrylamide gels. The sizes of the resulting PCR products and the groups that they identify are as follows: a 506-bp amplicon specific to the 16S rRNA gene for all mycobacteria; a 705-bp amplicon specific to *rv0577* for MTBC species; a 369-bp amplicon specific to RD9 for *M. tuberculosis*; a 231-bp amplicon specific to *mtbk_20680* for the *M. tuberculosis* Beijing family; a 600-bp amplicon specific to IS*1311* for MAC; a 106-bp amplicon specific to DT1 for *M. intracellulare*; a 310-bp amplicon and a 1,145-bp amplicon specific to *mass_3210* for *M. abscessus* and *M. massiliense*, respectively; and a 199-bp amplicon

TABLE 2 Identification of clinical isolates by standard genotyping or reference diagnostic method and the multiplex PCR assay

Clinical organism	No. of isolates tested	Standard genotyping for evaluation	Isolate origin
M. tuberculosis	39[a]/9[b]	Spoligotyping and commercialized real-time PCR method	Yonsei University, Wonju, South Korea[a]/Yonsei University College of Medicine, Seoul, South Korea[b]
M. avium	5[c]/5[b]	MLSA and commercialized real-time PCR method	Samsung Medical Center (SMC), Seoul, South Korea
M. intracellulare	5[c]/5[b]		
M. abscessus	5[c]/5[b]		
M. massiliense	5[c]/5[b]		
M. kansasii	5[c]/1[b]		
Total	94		

[a]Tested by spoligotyping.
[b]Tested by Advansure TB/NTM real-time PCR (LG Life Sciences, Daejeon, South Korea).
[c]Tested by MLSA (multilocus sequencing analysis).

specific to *mkan_rs12360* for *M. kansasii* (Table 1). For preliminary testing of the multiplex PCR, we included the eight main *Mycobacterium* species and also used one NTM species (*M. fortuitum*) and one nonmycobacterial species (*Escherichia coli*) as negative controls. No nonspecific amplification bands were seen in any of the multiplex PCR products, and the assay correctly identified the reference strains, as shown in Fig. S1 and Table S1 in the supplemental material.

Development and evaluation of the first multiplex PCR assay. We evaluated a total of 108 strains, comprising 64 clinical isolates (39 *M. tuberculosis* and 25 NTM) (Table 2) and 44 reference strains (9 MTBC, 34 NTM, and 1 nonmycobacterial species) (Table 3). Clinical isolates were previously analyzed by spoligotyping (39 *M. tuberculosis* isolates [see Table S2 in the supplemental material]) and multilocus sequence analysis (MLSA) (25 NTM, data not shown) for comparison with our multiplex PCR results (all isolates). Among the 64 clinical isolates that were analyzed by spoligotyping and MLSA, 25 Beijing family and 14 non-Beijing family species were found in the *M. tuberculosis* clinical isolates, and 5 each of *M. avium*, *M. intracellulare*, *M. abscessus*, *M. massiliense*, and *M. kansasii* were found in the NTM clinical isolates. Compared with our multiplex PCR results, all clinical isolates except two (MTB067 and 0A077) were correctly matched. According to standard genotyping (Table S2), the MTB067 and 0A077 isolates belong to the *M. tuberculosis* Central Asia strain (CAS) lineage (lineage 3), which is a non-Beijing family of *M. tuberculosis*. However, they were misidentified as members of the *M. tuberculosis* Beijing family (lineage 2 of *M. tuberculosis*) by displaying the positive amplicon for *mtbk_20680* in the initial design of our multiplex PCR assay. The *M. tuberculosis* Beijing genotype and CAS family are major lineages in Asian countries but occupy different geographical locations (21). In addition, they are closer on the nucleotide level than other *M. tuberculosis* lineages when analyzed by spoligotyping. When using spoligotype-based lineage identification, *M. tuberculosis* Beijing family isolates are sometimes classified as belonging to *M. tuberculosis* CAS (10, 22, 23). Although we wanted to increase the number of isolates to better evaluate the potential of our assay to discriminate between *M. tuberculosis* CAS and *M. tuberculosis* Beijing family strains in clinical isolates, we could not obtain more CAS isolates.

The 108 clinical and reference strains were correctly identified at the lineage and species levels with our multiplex PCR assay, except for the two strains described above. The assay demonstrated 100% sensitivity and 94.8% specificity for *M. tuberculosis*. In addition, the assay yielded 100% sensitivity and 100% specificity for MTBC and NTM.

Optimization of the multiplex PCR assay. Based on our *in silico* analysis system, the CAS/NITR 204 strain, which was used as a reference strain for the *M. tuberculosis* CAS

TABLE 3 Reference strains and clinical isolates evaluated in the multiplex PCR assay

Species	Source[a] or designation	No. of isolates tested	Target amplicon								
			16S rRNA gene	*rv0577*	RD9	*mtbk_20680*	IS*1311*	DT1	*mass_3210*	*mkan_rs12360*	RD750
Mycobacterium tuberculosis complex											
M. tuberculosis Beijing family	HN878, K	2	+	+	+	+	−	−	−	−	+
M. tuberculosis non-Beijing family	H37Rv ATCC 27294 H37Ra ATCC 25177	2	+	+	+	−	−	−	−	−	+
M. africanum, type 1	ATCC 25420	1	+	+	−	−	−	−	−	−	+
M. bovis	ATCC 19210, AN5	2	+	+	−	−	−	−	−	−	+
M. bovis BCG	BCG Pasteur 1173P2	1	+	+	−	−	−	−	−	−	+
M. microti	ATCC 19422	1	+	+	−	−	−	−	−	−	+
Targeted nontuberculous mycobacteria											
M. avium	ATCC 700898	1	+	−	−	−	+	−	−	−	−
M. intracellulare	ATCC 13950	1	+	−	−	−	−	+	−	−	−
M. abscessus	ATCC 19977	1	+	−	−	−	−	−	+	−	−
M. massiliense	CIP 108297	1	+	−	−	−	−	−	+	−	−
M. kansasii	ATCC 12478	1	+	−	−	−	−	−	−	+	−
Other nontuberculous mycobacteria											
M. aichiense	ATCC 27280	1	+	−	−	−	−	−	−	−	−
M. asiaticum	ATCC 27276	1	+	−	−	−	−	−	−	−	−
M. aurum	ATCC 23366	1	+	−	−	−	−	−	−	−	−
M. austroafricanum	ATCC 33464	1	+	−	−	−	−	−	−	−	−
M. avium	ATCC 25291	1	+	−	−	−	+	−	−	−	−
M. celatum	ATCC 52231	1	+	−	−	−	−	−	−	−	−
M. chelonae	ATCC 35749	1	+	−	−	−	−	−	−	−	−
M. fortuitum	ATCC 49404	1	+	−	−	−	−	−	−	−	−
M. gastri	ATCC 15754	1	+	−	−	−	−	−	−	−	−
M. genavense	ATCC 51233	1	+	−	−	−	−	−	−	−	−
M. gordonae	ATCC 14470	1	+	−	−	−	−	−	−	−	−
M. hassiacum	ATCC 700660	1	+	−	−	−	−	−	−	−	−
M. interjectum	ATCC 51457	1	+	−	−	−	−	−	−	−	−
M. kubicae	ATCC 700732	1	+	−	−	−	−	−	−	−	−
M. malmoense	ATCC 29571	1	+	−	−	−	−	−	−	−	−
M. marinum	ATCC 927	1	+	−	−	−	−	−	−	−	−
M. neoaurum	ATCC 25795	1	+	−	−	−	−	−	−	−	−
M. nonchromogenicum	ATCC 19530	1	+	−	−	−	−	−	−	−	−
M. peregrinum	ATCC 14467	1	+	−	−	−	−	−	−	−	−
M. phlei	ATCC 11758	1	+	−	−	−	−	−	−	−	−
M. scrofulaceum	ATCC 19981	1	+	−	−	−	−	−	−	−	−
M. smegmatis	ATCC 9108	1	+	−	−	−	−	−	−	−	−
M. shimoidei	ATCC 27962	1	+	−	−	−	−	−	−	−	−
M. szulgai	ATCC 35799	1	+	−	−	−	−	−	−	−	−
M. terrae	ATCC 15755	1	+	−	−	−	−	−	−	−	−
M. thermoresistibile	ATCC 19527	1	+	−	−	−	−	−	−	−	−
M. triviale	ATCC 23292	1	+	−	−	−	−	−	−	−	−
M. vaccae	ATCC 15483	1	+	−	−	−	−	−	−	−	−
M. xenopi	ATCC 19250	1	+	−	−	−	−	−	−	−	−
Nonmycobacterial species											
Acinetobacter baumannii	ATCC 196060	1	−	−	−	−	−	−	−	−	−
Bacillus subtilis	KCTC 3068	1	−	−	−	−	−	−	−	−	−
Escherichia coli	ATCC PTA-4750, K1, ATCC 35218	3	−	−	−	−	−	−	−	−	−
Klebsiella pneumoniae	ATCC 13883	1	−	−	−	−	−	−	−	−	−
Pseudomonas aeruginosa	ATCC 47085D-5	1	−	−	−	−	−	−	−	−	−
Salmonella enterica serovar Enteritidis	ATCC 13076	1	−	−	−	−	−	−	−	−	−
Salmonella enterica serovar Typhimurium	ATCC 53648	1	−	−	−	−	−	−	−	−	−
Staphylococcus aureus	ATCC BAA-1556	1	−	−	−	−	−	−	−	−	−

(Continued on next page)

TABLE 3 (Continued)

Species	Source[a] or designation	No. of isolates tested	Target amplicon								
			16S rRNA gene	*rv0577*	RD9	*mtbk_20680*	IS*1311*	DT1	*mass_3210*	*mkan_rs12360*	RD750
Staphylococcus epidermidis	KCTC 1917	1	–	–	–	–	–	–	–	–	–
Streptococcus pneumoniae	ATCC 49619	1	–	–	–	–	–	–	–	–	–
Total		55									

[a]CIP, Collection of the Pasteur Institute, France; KCTC, Korean Collection for Type Cultures, Dajeon, South Korea.

lineage (24), did not have the *M. tuberculosis* Beijing-specific gene cluster *mtbk_20620-mtbk_20700*. However, spoligotyping results (Table S2) identified MTB067 and A077 as belonging to the *M. tuberculosis* CAS lineage, which conflicted with results obtained by the multiplex PCR assay. Therefore, we reorganized and reoptimized the multiplex PCR assay to better differentiate the *M. tuberculosis* Beijing family from other *M. tuberculosis* lineages.

To identify *M. tuberculosis* CAS exceptions, we replaced the *rv0577* primer pair with another one that could discriminate between MTBC and NTM and could also distinguish *M. tuberculosis* CAS from other *M. tuberculosis* lineages. The target gene region was RD750 (*rv1519-rv1520*), which is an *M. tuberculosis* CAS-specific deletion (21). The predicted PCR product size was 865 bp (primer set 9 in Table 1). Optimization results for the multiplex PCR assay are shown in Fig. 2 and Table 4. We compared clinical *M. tuberculosis* isolate results of the first multiplex PCR with those of the second multiplex PCR (Fig. 3; see also Fig. S2 in the supplemental material). If a sample yielded amplicons of 506 bp (16S rRNA gene) and 369 bp (RD9) in the optimized PCR assay, we concluded that the isolate was of the *M. tuberculosis* CAS lineage because, as with CAS/NITR 204, it lacked the *M. tuberculosis* Beijing family-specific region. With the exception of *rv0577*, the compositions of the first and second primer sets for detecting NTM were exactly the same, so the band pattern and product sizes were also the same (Fig. 3B). In addition, we tested the specificity of our primers for mycobacterial 16S rRNA against 10 nonmycobacterial species (12 strains), and none of the tested bacteria produced detectable amplicons in the multiplex PCR (Table 3). This optimization assay demonstrated 100% sensitivity and 100% specificity for *M. tuberculosis*. In addition, the assay yielded 100% sensitivity and 100% specificity for both MTBC and NTM.

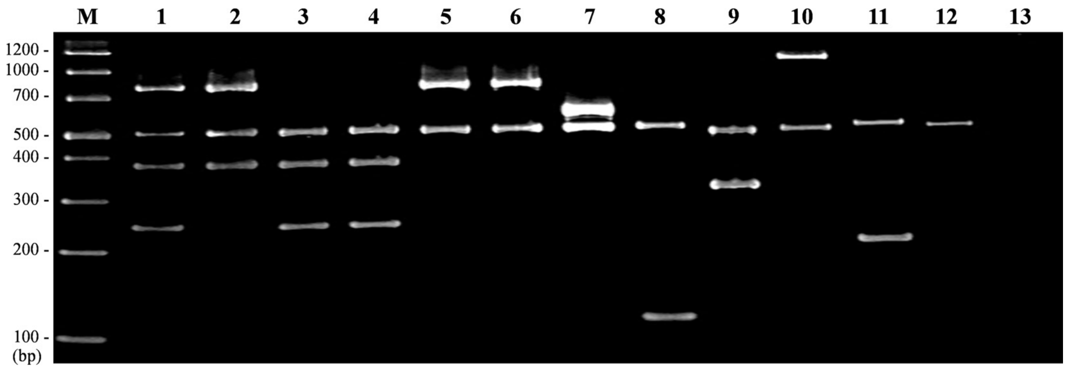

FIG 2 Representative multiplex PCR results after optimization to discriminate between the *M. tuberculosis* Beijing family and CAS lineage. The *rv0577* primer set was replaced with the RD750 primer set in the multiplex PCR assay to discriminate between the *M. tuberculosis* Beijing and CAS lineage. Lane M, molecular size marker; lane 1, *M. tuberculosis* HN878 (Beijing family); lane 2, *M. tuberculosis* H37Rv (non-Beijing family); lane 3, MTB067 (clinical CAS); lane 4, 0A077 (clinical CAS); lane 5, *M. bovis* BCG Pasteur 1173P2; lane 6, *M. africanum* ATCC 25420; lane 7, *M. avium* subsp. *hominissuis* 104; lane 8, *M. intracellulare* ATCC 13950; lane 9, *M. abscessus* ATCC 19977; lane 10, *M. massiliense* CIP 108297; lane 11, *M. kansasii* ATCC 12478; lane 12, *M. fortuitum* ATCC 49403; lane 13, *Escherichia coli* ATCC 35218.

TABLE 4 Interpretation criteria for the optimized multiplex PCR results

Organism identification or isolate designation	Criterion								No. of bands
	16S rRNA gene	RD750[a]	RD9	*mtbk_20680*	IS*1311*	DT1	*mass_3210*	*mkan_rs12360*	
M. tuberculosis HN878	+	+	+	+	−	−	−	−	4
M. tuberculosis H37Rv	+	+	+	−	−	−	−	−	3
MTB067[b]	+	−	+	+	−	−	−	−	3
0A077[b]	+	−	+	+	−	−	−	−	3
M. bovis BCG	+	+	−	−	−	−	−	−	2
M. africanum	+	+	−	−	−	−	−	−	2
M. avium	+	−	−	−	+	−	−	−	2
M. intracellulare	+	−	−	−	−	+	−	−	2
M. abscessus	+	−	−	−	−	−	+	−	2
M. massiliense	+	−	−	−	−	−	+	−	2
M. kansasii	+	−	−	−	−	−	−	+	2
M. fortuitum[c]	+	−	−	−	−	−	−	−	1
Escherichia coli	−	−	−	−	−	−	−	−	0

[a]RD750 was used in place of *rv0577* to discriminate between the CAS and Beijing family (see Fig. S1 and S2 in the supplemental material).
[b]Two clinical isolate exceptions (CAS) in the first multiplex PCR assay.
[c]*M. fortuitum* was used as the representative template for another NTM species.

Next, we examined the limit of detection for the optimized multiplex PCR using serially diluted DNA from the targeted *Mycobacterium* spp. (Fig. 4). The analytical sensitivity of the PCR ranged from 1 to 10 pg for all tested *Mycobacterium* spp., which is considered to be approximately equivalent to 10^3 to 10^4 CFU of mycobacteria (Fig. 4).

Evaluation of the optimized multiplex PCR for clinical applicability. We further investigated whether the multiplex PCR assay can be employed in a clinical setting by using a predetermined number of isolates of the targeted *Mycobacterium* spp. The sensitivity of the multiplex PCR using DNA directly extracted from 10^2 to 10^7 mycobacteria was compared with that using DNA extracted from the same number of mycobacteria mixed with nonhomogenized artificial sputum samples (Fig. 5). Consistent with the results of analytical sensitivity (Fig. 4), DNA directly extracted from 10^3 to

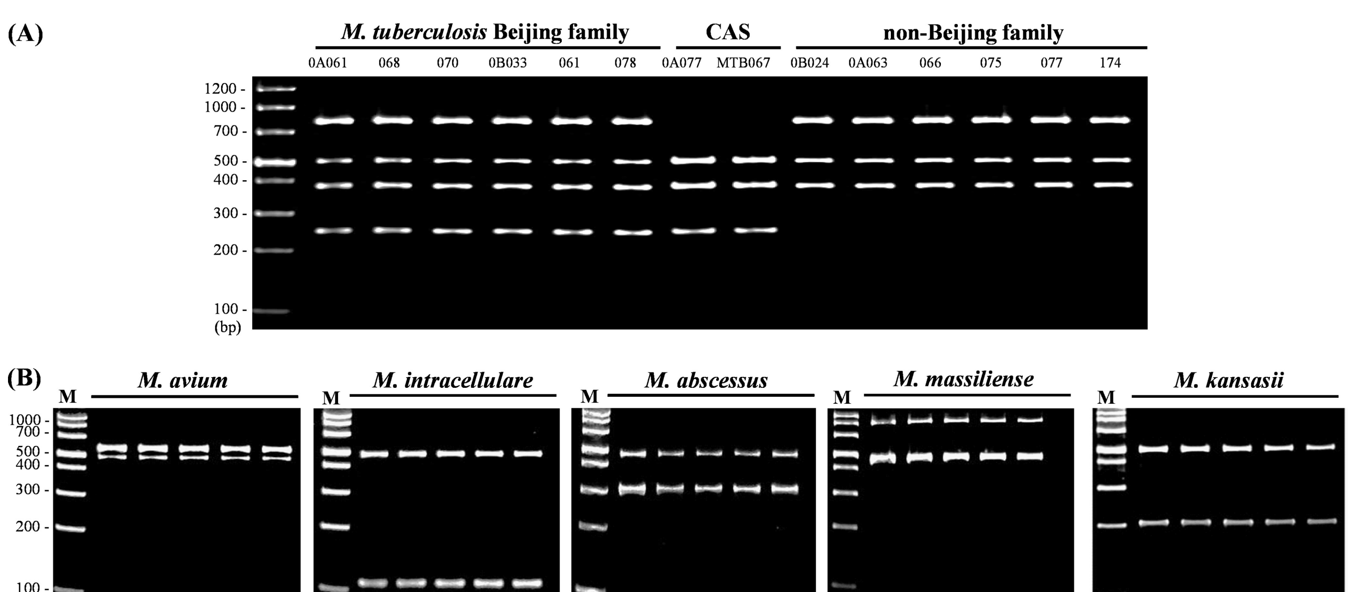

FIG 3 Optimized multiplex PCR results of *M. tuberculosis* and five major nontuberculous mycobacterial (NTM) clinical isolates. All clinical strains had previously been analyzed by spoligotyping for *M. tuberculosis* and multilocus sequencing analysis for NTM species. Six *M. tuberculosis* Beijing family strains, two *M. tuberculosis* CAS lineage strains, six *M. tuberculosis* non-Beijing family strains, and five of each targeted NTM species were selected. (A) Optimized multiplex PCR with *M. tuberculosis* clinical isolates. The multiplex PCR could discriminate *M. tuberculosis* Beijing family strains, *M. tuberculosis* CAS lineage strains, and *M. tuberculosis* non-Beijing family strains (with the exception of the *M. tuberculosis* CAS lineage) in *M. tuberculosis*. (B) Optimized multiplex PCR with NTM clinical isolates. All target NTM species yielded a 506-bp 16S rRNA gene amplicon. Each NTM species also yielded an amplicon specific to that NTM species.

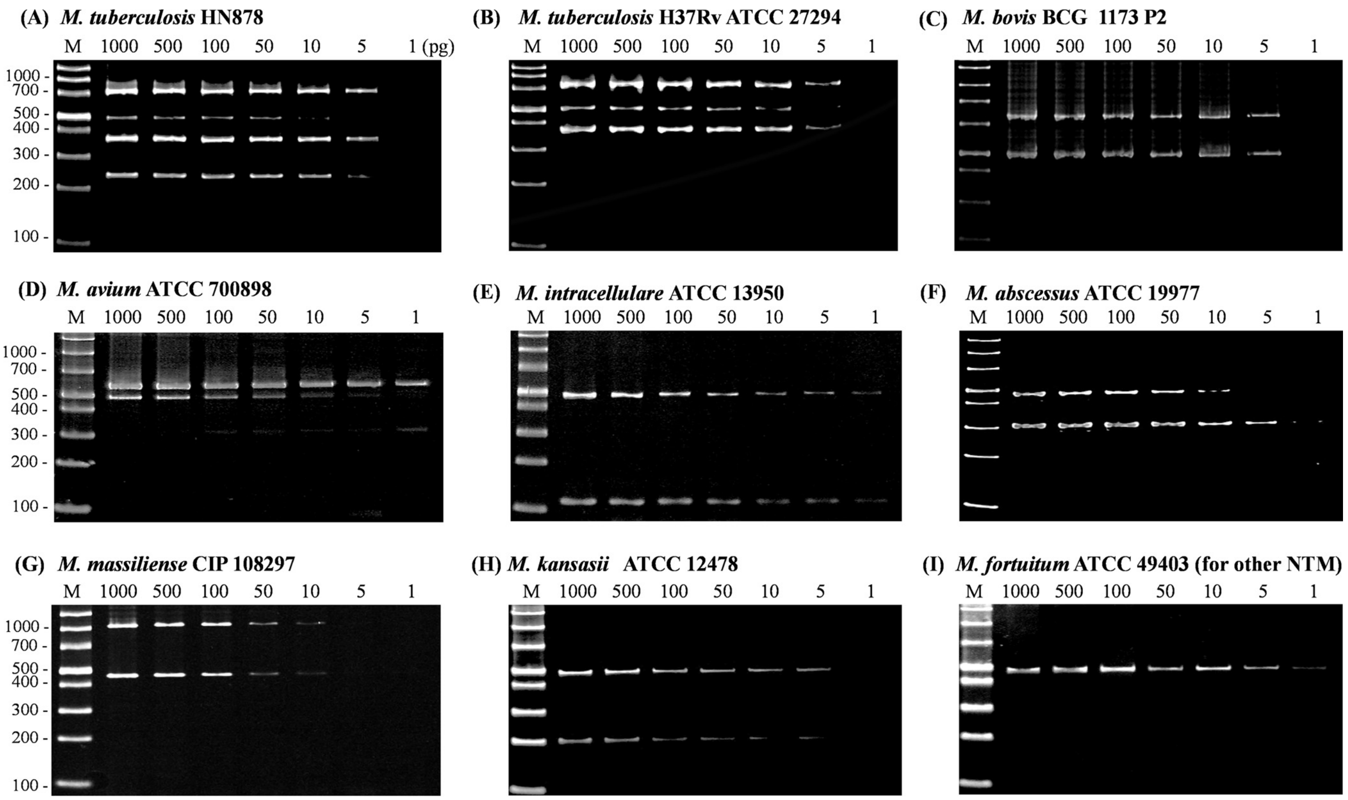

FIG 4 Analytical sensitivity of the multiplex PCR according to DNA amount. Sensitivity was evaluated for the targeted *Mycobacterium* species using 1 to 1,000 pg DNA per reaction mixture. The analytical sensitivities were 10 pg of *M. tuberculosis* HN878 (Beijing family) DNA (A), 10 pg of *M. tuberculosis* H37Rv (non-Beijing family) DNA (B), 5 pg of *M. bovis* BCG DNA (C), 10 pg of *M. avium* DNA (D), 1 pg of *M. intracellulare* DNA (E), 10 pg of *M. abscessus* DNA (F), 10 pg of *M. massiliense* (G), 5 pg of *M. kansasii* DNA (H), and 1 pg of *M. fortuitum* DNA (chosen to represent other NTM) (I).

10^4 mycobacterial cells in pure culture was detectable, whereas 10-fold more mycobacteria (ranging from 10^4 to 10^5) were required for the multiplex PCR detection with nonhomogenized artificial sputum samples (Fig. 5). Next, in a blinded manner, we clinically evaluated the assay using 30 sputum sample cultures grown in mycobacteria growth indicator tubes (MGIT) by comparing the results with those obtained with a commercial real-time PCR assay. The multiplex PCR showed 100% identification agreement with the commercial assay (Fig. 6).

Next, we investigated whether the multiplex PCR assay could accurately identify *M. tuberculosis* in the presence of NTM, an important consideration when TB patients are also infected with NTM. In this experiment, the amount of *M. tuberculosis* DNA was fixed to 100 pg and then mixed with 100-fold-lower to 100-fold-greater amounts of DNA from NTM species (see Fig. S3 in the supplemental material). Not only could the multiplex PCR assay differentially detect each *Mycobacterium* spp., but there was also no interference in detection, except when DNA from the *M. tuberculosis* Beijing strain was mixed with excess DNA from *M. intracellulare* (a >10-fold-greater amount) (Fig. S3).

DISCUSSION

The development of a method for efficiently and differentially identifying MTBC and the major NTM organisms causing human disease has the potential to greatly aid the control of mycobacterial infections. Most importantly, the increased possibility for accurate and early identification of specific species or subspecies can improve patient management, decisions on therapeutic regimens, and public health. In the present study, we developed a simple and effective one-step multiplex PCR assay that enables the differential identification of medically important *Mycobacterium* species as well as subspecies/members of the major mycobacterial complexes, including MTBC, MAC, and

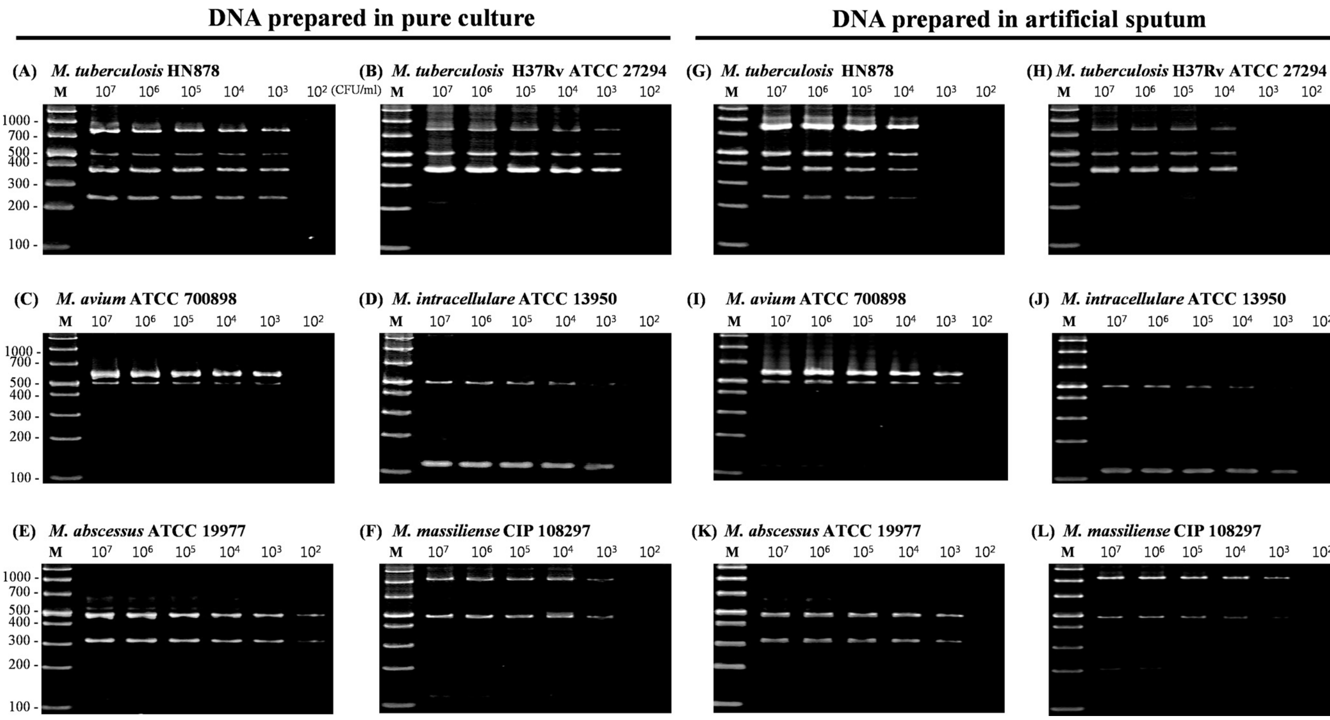

FIG 5 Measurement of the multiplex PCR sensitivity based on the numbers of bacteria in homogenized pure culture and nonhomogenized artificial sputum. DNA was extracted from the purely cultured bacteria (A to F) or from bacteria that were mixed with nonhomogenized artificial sputum (G to L). The abscissa represents the number of bacteria per milliliter.

MABC. The specific aims of the assay were to (i) detect all *Mycobacterium* species, (ii) differentiate between MTBC and NTM, (iii) differentiate between *M. tuberculosis* and MTBC, (iv) selectively identify the *M. tuberculosis* Beijing family among *M. tuberculosis* members, and (v) simultaneously identify five clinically important NTM species causing lung disease.

In clinical laboratories, various methods for identifying *Mycobacterium* species have been introduced over the past decades (25). Biochemical tests and high-performance liquid chromatography have been surpassed by molecular techniques as the methods of choice for discriminating the species of this genus. Molecular techniques, including restriction fragment length polymorphism analysis, spoligotyping, variable-number tandem repeat assays, PCR-based assays, and whole-genome sequencing (26), have been used to detect and identify mycobacterial species. These techniques reduce the turnaround time for the results to be reported, thereby enabling the acceleration of treatment decisions according to the etiologic species (26–29). Multiplex PCR assays for the identification of specific groups of mycobacteria, mainly MTBC and MAC,

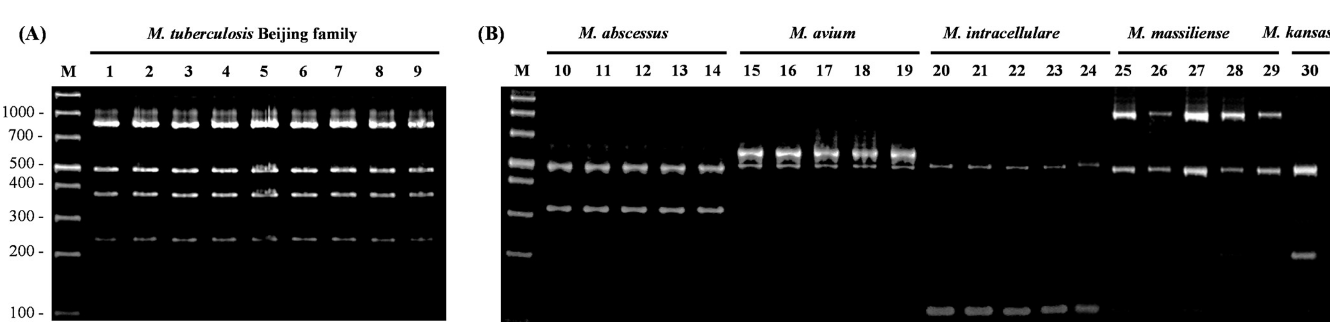

FIG 6 Multiplex PCR assay with *M. tuberculosis* and NTM cultured in Bactec MGIT-960 from patient sputa. Species were previously identified by AdvanSure TB/NTM real-time PCR. (A) Results of the multiplex PCR assay with *M. tuberculosis*. (B) Results of the multiplex PCR assay with NTM. Result of the multiplex PCR assay showed 100% agreement with the commercial real-time PCR assay.

have been developed and evaluated (15, 17, 18, 29, 30). However, although several multiplex PCR systems have already been commercialized and introduced into laboratories (30–32), to the best of our knowledge, there are no one-step multiplex PCR assays capable of simultaneously discriminating various clinically important major *Mycobacterium* species. Moreover, established PCR assays also normally require two PCR steps to distinguish the mycobacterial isolates to the species and/or subspecies level (33, 34).

In this study, we developed a new multiplex PCR assay that simultaneously targets major *Mycobacterium* species causing human disease and that has a broad detection range. Insertion or deletion regions specific to each species of *Mycobacterium* provided meaningful markers for their identification. All primers, except for RD9, were newly designed and optimized for development of the multiplex PCR assay in this study.

For verification of mycobacterial identification, amplification of the 16S rRNA gene served as an internal control (15). Then, based on *rv0577*, we classified all tested mycobacteria as either MTBC or NTM. In general, *rv0577* and IS*6110* can be used to detect MTBC (15, 35). However, although IS*6110* can be distributed throughout the whole MTBC genome, some MTBC strains have few or no copies of this element (35), and therefore *rv0577* may be a more reliable target than IS*6110*. Another of our targets, RD9, can be used to differentiate *M. tuberculosis* from the other MTBC species (17) because it is missing from all MTBC species except *M. tuberculosis*. Our assay also includes primer sets for five target NTM genes: IS*1311* for *M. avium*, DT1 for *M. intracellulare*, *mass_3210* for *M. abscessus* and *M. massiliense*, and *mkan_rs12360* for *M. kansasii*. In addition, *mass_3210* was newly employed in this study, and all of the NTM-targeting primer sets have been used as reliable markers for their corresponding species. Lastly, *mtbk_20680* is used to target the *M. tuberculosis* Beijing family.

The data presented here suggest several limitations of this multiplex PCR assay. First, two *M. tuberculosis* clinical isolates belonging to the CAS lineage were identified as belonging to the *M. tuberculosis* Beijing family by *mtbk_20680* detection. Of note, the CAS lineage and Beijing family are much closer by single nucleotide polymorphism (SNP) type and spoligotyping than other lineages. For example, the *M. tuberculosis* CAS lineage belongs to principal genetic group 1 (PGG1) and cluster II, which includes the East-Africa-Indian and East-Asian lineages (21). PGG1 strains usually share spacers 35 and 36, which are lacking in other strains, and the *M. tuberculosis* CAS lineage and *M. tuberculosis* Beijing family have many more overlapping SNPs than other lineages. Therefore, these lineages are sometimes classified into the same (or a similar) genotype family (21). It has also been reported that pseudo-Beijing strains harbor the Beijing spoligotyping patterns, but these strains have smaller deletions in RD207 than those of true Beijing strains (36). Since few genomic data have been published for the CAS lineage, we selected *mtbk_20680* based on genomic analysis of CAS/NITR204 for lineage 3, which belongs to PGG1. Genome information for this lineage is available only among CAS lineages in the public databases. In addition, one CAS strain was allocated to the Beijing family when its whole-genome sequence was analyzed by average nucleotide identity (data not shown), whereas this strain demonstrated CAS patterns in spoligotyping assays. Thus, *mtbk_20680* is uniquely conserved in the Beijing lineage and in CAS genetically close to the Beijing family; the *mtbk_20680*-associated cluster may be useful for understanding evolutionary processes and in epidemiological studies of the Beijing lineage and related genotypes.

A second limitation of our multiplex PCR assay is its inability to differentiate *M. canettii* from the *M. tuberculosis* Beijing family. However, although the multiplex PCR patterns were identical for *M. canettii* and the *M. tuberculosis* Beijing family (data not shown), *M. canettii* is extremely rare in human cases, with most cases found in the Horn of Africa, and it displays a distinct smooth morphotype (2) that can also be used to aid identification. *M. canettii* is considered a phylogenetic prototype of MTBC and evolutionally located outside the main subspecies of MTBC (2). For these reasons, clinical identification of *M. canettii* is ruled out occasionally when developing molecular diagnostic tests for *Mycobacterium* spp. (2, 37).

Third, IS*1311* and DT1 are reliably found in *M. avium* and *M. intracellulare*, respectively, with only one exception reported (18). IS*1311* alone may be not enough to classify all isolates to the MAC subspecies level. Although *M. avium* subsp. *hominissuis* is the major member of the MAC that infects humans, other MAC subspecies (including *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*), which generally cause animal disease, can also cause human disease (18). Recently, Kim et al. reported that although DT1 is thought to be specific to *M. intracellulare*, it has occasionally been observed in *M. avium*. Since this element is mobile by nature, it is possible to find this element in related or unrelated bacteria (38). Thus, strains known to be exceptions should be tested using our multiplex PCR assay.

Fourth, *M. massiliense* and *M. bolletii* were recognized as the same species. However, *M. bolletii* was recently reported to have a taxonomic status independent from *M. massiliense* (39). Although *M. bolletii* is an MABC that rarely causes human disease, it would be preferable if the multiplex PCR mixture contained a marker to discriminate these three subspecies within the MABC, including *M. bolletii*.

Fifth, mixed infection of *M. tuberculosis* and NTM is difficult to diagnose in clinical practice (40). Our multiplex PCR assay has the advantage of being able to detect mixed-infection cases, including *M. tuberculosis* and NTM (see Fig. S3 in the supplemental material). However, it is difficult to discriminate coinfections with *M. tuberculosis* Beijing family and non-Beijing family species, since the multiplex PCR assay results are obtained by visual readout.

Sixth, for the initial evaluation on the optimized multiplex PCR assay, we used high-quality DNA manually prepared from carefully controlled cultures of each of the five clinical isolates of NTM species and subspecies and 64 MTBC (Fig. 3). Although the multiplex PCR was able to identify MGIT cultures even after a crude DNA preparation method such as boiling (Fig. 6), the analytical sensitivity of the assay to nonhomogenized artificial sputum samples was 10-fold lower than to purely cultured bacteria (Fig. 5). Thus, further testing is required to determine its suitability for identifying species directly in acid-fast bacillus smear-positive respiratory specimens such as human sputum samples.

Nevertheless, additional advantages of our assay are that it requires only common PCR reagents, uses a simple method, and employs standard equipment; these features frequently underpin routine diagnostic tests in laboratories. In contrast, real-time PCR requires expensive equipment and reagents. In addition, our one-step multiplex PCR platform does not require an additional step (e.g., a nested-PCR platform). Thus, the assay reliability is relatively high compared to other assays, since sample contamination is reduced. In addition, the primer sets adopted in this study can be easily combined according to the targeted species and the study purpose. In conclusion, our one-step multiplex PCR platform is a convenient, cost-effective, and reliable method for detecting the most clinically important *Mycobacterium* species. The data provided from the multitarget PCR assay allow discrimination among major mycobacteria causing lung disease and improve diagnostic confidence in the identity of isolates. This assay will potentially improve the discrimination of clinical *Mycobacterium* isolates and also promises to be useful for the development of a routine method to be used in the clinical setting.

MATERIALS AND METHODS

Bacterial collection and identification of *Mycobacterium* species. In this study, we tested 55 reference strains consisting of 8 MTBC strains (2 *M. tuberculosis* Beijing family strains, HN878 [41] and strain K [42], 2 *M. tuberculosis* non-Beijing family strains, *M. bovis strains*, *M. bovis* BCG, *M africanum*, and *M. microti*), 34 NTM ATCC strains, and 12 nonmycobacterial strains (Table 3). We also tested 94 clinical strains (48 *M. tuberculosis* and 46 NTM isolates) (Table 2). Genotyping of the 39 *M. tuberculosis* and 9 *M. tuberculosis* clinical isolates was determined, respectively, by spoligotyping, which is a gold standard genotyping assay, and Advansure TB/NTM real-time PCR (LG Life Sciences, Daejeon, South Korea), a commercial kit used for diagnosis in laboratory medicine (4). Spoligotyping of the 39 *M. tuberculosis* isolates was performed at Yonsei University (Wonju, South Korea), and real-time PCR of the 9 *M.*

tuberculosis strains was performed at Yonsei University (Seoul, South Korea). In addition, the 25 NTM and 21 NTM clinical strains were identified by MLSA and Advansure TB/NTM real-time PCR at Samsung Medical Center (Seoul, South Korea) (19, 43–45).

Bacterial cultures and DNA preparation. Mycobacterial culture was carried out as previously described (18). Briefly, mycobacteria were cultivated in Middlebrook 7H9 liquid medium (pH 6.8; Difco Laboratories, Detroit, MI, USA) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC; BD Diagnostics, Sparks, MD, USA) and incubated at 37°C or 30°C. Mycobacterial DNA from controlled cultures was extracted using the conventional cetyltrimethylammonium bromide method according to procedures previously described, with slight modifications (46). DNA preparations from Bactec MGIT-960 tubes were performed using a Qiagen DNeasy blood & tissue kit (Qiagen, Inc., Valencia, CA, USA) or a simple boiling method (47).

Identification of *Mycobacterium* species-specific target sequences and primer design. We employed two analytical strategies for the determination of targets and development of the multiplex PCR assay: (i) comparative genomic analysis of MTBC and MABC and (ii) bioinformatics analysis of *M. avium*, *M. intracellulare*, and *M. kansasii* based on previous studies and data available in the public domain via NCBI (http://www.ncbi.nlm.nih.gov/). Comparative genomic analysis was performed to find specific target sequences for the *M. tuberculosis* Beijing family and MABC strains. We selected *M. tuberculosis* K (GenBank accession no. CP007803.1) as the representative *M. tuberculosis* strain for the Beijing family (42) and *M. abscessus* (accession no. CU458896) and *M. massiliense* (accession no. NZ_JRMG00000000.1) as the representative MABC strains (19). On alignment of the complete genomes of the K and H37Rv ATCC 27294 strains (accession no. NC_000962), several polymorphic sites that corresponded to single genes or insertion or deletion regions were found. The polymorphic regions were also compared with those of other strains of the Beijing family, including *M. tuberculosis* HN878, CCDC5079, CCDC5180, CTRI-4, 210, NCGM2209, R1207, and X122, and non-Beijing family strains, including EAI5/NITR206 for lineage 1, CAS/NITR204 for lineage 3, CDC1551 and F11 for lineage 4, ATCC 25420 for lineage 5, and GM 041182 for lineage 6, from previous publications and clinical isolates (24).

For MABC-specific target sequences, the *M. massiliense* complete genome was compared with that of *M. abscessus* (19), and several different regions were analyzed according to the steps described above. The other sequences (16S rRNA gene [15], *rv0577* [15], RD9 [17], IS*1311*, DT1 [18], and *mkan_rs12360* [20]) were selected based on previous publications and bioinformatics analysis.

PCR primers were designed based on each target sequence, using the Vector NTI program (Thermo Fisher Scientific, MA, USA), and further analyzed to predict primer-dimer interactions when mixed in a multiplex PCR. Primer design for the 16S rRNA gene, *rv0577* (15), RD9 (*rv2072*c and *rv2073*c) (17), and RD750 (*rv1519* and *rv1520*) (15) amplicons was based on the *M. tuberculosis* H37Rv strain ATCC 27294. Primer pairs were designed based on the sequences of *mtbk_20680* in *M. tuberculosis* strain K, IS*1311* in *M. avium* (accession no. NC_008595), DT1 in *M. intracellulare* (accession no. NC_016946) (18), and *mkan_rs12360* in *M. kansasii* (accession no. NC_022663) (20). *mab_3265*c in *M. abscessus* was formerly referred to as *mass_3210*, which is a single gene specific to *M. massiliense*. The *M. abscessus* and *M. massiliense* target primer pair was created using the region flanking *mass_3210* (*mab_3265*c/*mass_3209* to *mab_3266*c/*mass_3211*) (Table 1).

Multiplex PCR amplification and assay interpretation criteria. For amplification, each PCR mixture contained 25 μl of 2× EF-*Taq* PCR Smart mix (Solgent Co., Ltd. Daejeon, South Korea), 9.2 μl of primer mix containing 4 μl of the *rv0577* primer set, 0.3 μl of the DT1 and *mtbk_20680* primer sets, and 1 μl of the 16S rRNA, RD9, IS*1311*, *mass_3210*, and *mkan_rs12360* primer sets (all primer solutions in 10 pmol), 2 μl of DNA template, and 12.8 μl of water in a final volume of 50 μl. PCR amplification was performed in an S1000 Thermal cycler. Thermocycling parameters included an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 96°C for 45 s, 61.5°C for 45 s, and 72°C for 40 s. The final extension step was performed at 72°C for 10 min. After amplification, PCR products were analyzed via acrylamide gel electrophoresis on 6% acrylamide gels and stained with 1 μl of Gel Star (Lonza, Basel, Switzerland) in 50 ml of 1× Tris-borate-EDTA (TBE) buffer. The assay products were electrophoresed for 37 min at 120 V in 1× TBE buffer, and the gels were visualized under UV light.

The multiplex PCR interpretation criteria, which rely on deduction, and their target *Mycobacterium* species are listed in Table S1. Simultaneous amplification of the four targets *mtbk_20680*, RD9, *rv0577*, and 16S rRNA gene was interpreted as association with the *M. tuberculosis* Beijing family; amplification of three targets excluding *mtbk_20680* was interpreted as association with *M. tuberculosis* non-Beijing family species; and amplification of two targets excluding *mtbk_20680* and RD9 was interpreted as association with MTBC. Amplification of IS*1311*, DT1, or *mkan_rs12360* with the 16S rRNA gene was interpreted as identifying with *M. avium*, *M. intracellulare*, or *M. kansasii*, respectively, and amplification of *mass_3210* with the 16S rRNA gene was interpreted as identifying with *M. abscessus* and *M. massiliense*. *Mycobacterium* species outside the multiplex PCR target species were indicated by observation of only 16S rRNA gene amplification.

Optimization of the multiplex PCR assay. The accuracy (specificity and sensitivity) of the multiplex PCR assay was evaluated using the 64 *M. tuberculosis* clinical isolates described above (Table 2). The PCR products of all clinical strains were confirmed and compared with spoligotyping (Table S2) and MLSA results (NTM PCR results are shown in Fig. 3B).

After the initial evaluation, *rv0577* was replaced with RD750. Multiplex PCR optimization was then performed under the same PCR amplification conditions as described above, with the exception of the primer composition. Specifically, the primer mixture (7.6 μl) consisted of 2 μl of RD9, 1 μl of DT1 and RD750, 0.8 μl of 16S rRNA, IS*1311*, and *mass_3210*, and 0.6 μl of the *mtbk_20680* and *mkan_rs12360* primer sets (Table 1).

Analytical sensitivity of the multiplex PCR. For estimation of the limit of detection of the optimized multiplex PCR, we tested DNA from nine representative *Mycobacterium* species and lineage strains (*M. tuberculosis* HN878 [Beijing family], *M. tuberculosis* H37Rv ATCC 27294 [non-Beijing family], *M. bovis* BCG Pasteur 1173P2, *M. avium* ATCC 700898, *M. intracellulare* ATCC 13950, *M. abscessus* ATCC 19977, *M. massiliense* CIP 108297, *M. kansasii* ATCC 12478, and *M. fortuitum* ATCC 49404) with serial dilutions ranging from 100 ng to 0.01 pg per reaction mixture. After amplification, 4 μl of the PCR mixture was loaded onto 6% acrylamide gels. The detection limit was determined to be the smallest DNA quantity that generated the correct band pattern for the targeted *Mycobacterium* spp.

Evaluation of the optimized multiplex PCR assay for clinical applicability. The multiplex PCR assay was evaluated using DNA extracted from nonhomogenized artificial sputum, generated as previously described (48), that had been spiked with pure cultures of the targeted *Mycobacterium* spp. Mycobacterial DNA was prepared using a Qiagen kit as described above. Briefly, l ml of serial dilutions of the predetermined mycobacterial numbers was inoculated into 4 ml of 7H9-OADC broth and 4 ml of artificial sputum. The final CFU were adjusted to range from 10 to 10^8 CFU/sample for each tested *Mycobacterium* spp.

To assess applicability to coinfections of *M. tuberculosis* and NTM and possible interference in PCRs, the multiplex PCR was evaluated with mixed DNA from *M. tuberculosis* and each NTM species. Briefly, the amount of DNA from *M. tuberculosis* (HN878 for Beijing family and H37Rv for non-Beijing family) was fixed at 0.1 ng/reaction mixture, and DNA from each NTM species was added to the multiplex PCR mixture in increasing amounts to give *M. tuberculosis* DNA-to-NTM DNA ratios of 0.01 to 500.

Finally, the multiplex PCR was evaluated using DNA extracted from 9 *M. tuberculosis* and 21 NTM Bactec MGIT-960 cultures of patient sputa in a blinded manner. The identification results were compared with those obtained using AdvanSure TB/NTM real-time PCR. Since this real-time PCR kit is not able to discriminate between *M. abscessus* and *M. massiliense*, the presence of *erm*(41) was tested separately (19).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00549-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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